NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL

Division of Bacteriology

Standard Operating Procedure

Non lethal mouse local muscular paralysis assay:
In vivo assessment of botulinum type A toxin

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Introduction

Botulinum toxin type A induces muscular paralysis following specific binding and uptake by the pre-synaptic nerve terminal, and subsequent cleavage of SNAP25, a protein essential for the release of neurotransmitter at the synaptic junction. The non-lethal muscular paralysis assay relies on the measurement of the local flaccid muscular paralysis at the inguinocrural region of mice injected with a low dose of botulinum toxin (Takahashi *et al.* 1990; Sesardic *et al.* 1996). Toxin potency is reflected by the degree of muscular paralysis induced by the toxin. Activity of a toxin batch is estimated relative to a product specific toxin reference material of defined activity. The method is regulated under the UK Animal (Scientific procedures Act) 1986. It is the responsibility of the operator to ensure that the procedures they carry out are performed strictly in accordance with the method stated on the project licence and that their personal licence covers the techniques they are going to undertake.

Equipment:

class II EPC safety cabinet
sterile syringes 1ml
sterile needles 27G x 0.5" (0.4 x 12mm), and 21G x 1.5" (0.8 x 40mm)
pH meter
heater / stirrer
electronic balance
racks for tubes

Reagents and Chemicals:

Gelatin Phosphate Buffer, pH 6.5 (GPB)	
0.2% Gelatin	2g
0.05M Disodium hydrogen orthophosphate (2 hydrate)	8.9g
Water (Milli Q)	up to 1L

- Warm and stir the mixture until all the solids have dissolved.
- Allow to cool to room temperature and adjust the pH to 6.5 with 50% orthophosphoric acid and make up to 1L.
- Decant solution into 150ml glass bottles and sterilize by autoclaving at 121°C for 25 minutes. Store solution at +4°C.

Sample storage
Samples of botulinum type A toxin are stored according to the manufacturer's instructions. E.g. either in the fridge 4 to 8°C or freezer -15 to -45°C, as appropriate.

Animals:

Female mice, strain MF1 (Harlan) weighing 17-22g, are allowed to acclimatise according to in house standard procedures. Groups of 4 mice per cage are used for testing and each mouse is colour coded for identification. Animals are housed as indicated in the in house SOP for animal husbandry with free access to food and water.

Procedure:

Note – all work should be done in a class II EPC safety cabinet or a clean working area as appropriate.

Preparation of dilutions:

Using a 1ml syringe and needle (21G x 1.5") vials are carefully reconstituted with 1ml of GPB (Add GPB so that it strikes the wall of the vial first, to avoid vigorous agitation of the toxin) to give nominal potencies of 500U/ml assuming 500U/vial or 100U/ml assuming 100U/vial. Mix gently making sure that all the powder is dissolved and allow to stand at room for approximately 10 minutes.

Combine contents of duplicate vials using a fresh syringe and needle or after removing the vial stoppers and mix and dilute to 50U/ml (e.g. 100µl of 500U/ml nominal potency + 900µl GPB or 200µl of 100U/ml nominal potency + 200µl GPB).

A suitable range of dilutions should be made, e.g.:

Concentration U/ml	**Amount (ii) toxin µl**	**Amount GPB µl**	**Sample**
2.1	210	4790	REF, sample 1 (and 2)
1.5	150	4850	REF, sample 1 (and 2)
1.0	100	4900	REF, sample 1 (and 2)
0.5	50	4950	REF, sample 1 (and 2)

or

Concentration U/ml	**Amount (ii) toxin µl**	**Amount GPB µl**	**Sample**
1.5	150	4850	REF, sample 1 (and 2)
0.8	80	4920	REF, sample 1 (and 2)
0.4	40	4960	REF, sample 1 (and 2)
0.25	25	4975	REF, sample 1 (and 2)

Syringes (1ml with 27G x 0.5") are numbered and samples randomised before injection in two blocks of cages so that each dose of each preparation occurs once in each of the two blocks. Randomisation is carried out for each assay, and two examples of this randomization are shown below using one or two test samples.

Cage / syringe number	**Sample**	**Dose**	**Number of Mice**
1	-ve control GPB	0	4 per cage
2	REF	2.1 U/ml	4 per cage
6	REF	1.5 U/ml	4 per cage
9	REF	1 U/ml	4 per cage
7	REF	0.5 U/ml	4 per cage
4	Test Sample 1	2.1 U/ml	4 per cage
8	Test Sample 1	1.5 U/ml	4 per cage
5	Test Sample 1	1 U/ml	4 per cage
3	Test Sample 1	0.5 U/ml	4 per cage
17	REF	2.1 U/ml	4 per cage
12	REF	1.5 U/ml	4 per cage
16	REF	1 U/ml	4 per cage
14	REF	0.5 U/ml	4 per cage
10	Test Sample 1	2.1 U/ml	4 per cage
11	Test Sample 1	1.5 U/ml	4 per cage
13	Test Sample 1	1 U/ml	4 per cage
15	Test Sample 1	0.5 U/ml	4 per cage

Cage / syringe number	Sample	Dose	Number of Mice
1	-ve control GPB	0	4 per cage
2	REF	2.1 U/ml	4 per cage
12	REF	1.5 U/ml	4 per cage
6	REF	1 U/ml	4 per cage
7	REF	0.5 U/ml	4 per cage
4	Test Sample 1	2.1 U/ml	4 per cage
11	Test Sample 1	1.5 U/ml	4 per cage
5	Test Sample 1	1 U/ml	4 per cage
13	Test Sample 1	0.5 U/ml	4 per cage
3	Test Sample 2	2.1 U/ml	4 per cage
8	Test Sample 2	1.5 U/ml	4 per cage
9	Test Sample 2	1 U/ml	4 per cage
10	Test Sample 2	0.5 U/ml	4 per cage
17	REF	2.1 U/ml	4 per cage
25	REF	1.5 U/ml	4 per cage
20	REF	1 U/ml	4 per cage
21	REF	0.5 U/ml	4 per cage
18	Test Sample 1	2.1 U/ml	4 per cage
19	Test Sample 1	1.5 U/ml	4 per cage
22	Test Sample 1	1 U/ml	4 per cage
15	Test Sample 1	0.5 U/ml	4 per cage
24	Test Sample 2	2.1 U/ml	4 per cage
16	Test Sample 2	1.5 U/ml	4 per cage
14	Test Sample 2	1 U/ml	4 per cage
23	Test Sample 2	0.5 U/ml	4 per cage

Dosing and monitoring of animals:

Eight mice (2 cages of 4) receive an injection (s.c.) of 0.1ml volume of each dilution in the left inguinocrural region. Two operators are required to administer the injections, one person to hold the animal while the other carefully injects 0.1ml taking care not to inject too deeply.

Following injection of botulinum toxin, the animals are checked for signs of muscular paralysis at the inguinocrural / abdominal region at 24h and 48h post-injection (sometimes at 30h if required). Any animals showing slight signs of systemic toxicity should be more regularly monitored and culled immediately if moderate or severe signs of toxicity develop.

Scoring:

Scoring should be independently performed by trained individuals, preferably blind to the randomisation. Colour coded mice are picked up individually by their tail and scored independently by each observer.

0 : No signs, normal
1: Just detectable (slight bulge at injection site). E.g. Covering an area of approximately 0.5cm diameter or less (or less than two nipples).

2 : More pronounced bulge. E.g. Covering an area of greater than 0.5cm diameter (or greater than or equal to two nipples), but less than the maximum radius of the hind leg heal.
3: More extensive bulge extending over a larger area. Extending below hips / top of thigh when viewed from the side and beyond the maximum radius of the hind leg heal.
4: Maximal local effect. More extensive bulge extending over a larger area will often extend as far as the bottom of the rib cage, or over a large area with extensive distension or bulging.

'Beyond a 4'.: When high concentrations of toxin are used a No4 bulge may flatten out with time (e.g. at 48h) or go 'beyond a 4'. This may be an early (e.g. 24h) indicator of subsequent systemic signs of toxicity.

Examples of scoring forms are shown below. Additional sheets are used if additional times or other characteristics of the mice, such as weight, are recorded.

Title: <u>Botulinum toxin (type A) Non lethal assay</u> (PPL 80/_____) Date of assay: _____. Test ID number:_____
M1=Blue head, M2= Blue tail, M3=Pink head, M4=Pink tail.

Time Scored: _____ Observer Initials:_____

Time	Cage	M1 Blue H	M2 Blue T	M3 Pink H	M4 Pink T	Total score	Comment
24hr	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
	9						
	10						
	11						
	12						
	13						
	14						
	15						
	16						
	17						

Time Scored: _____ Observer Initials:_____

Time	Cage	M1 Blue H	M2 Blue T	M3 Pink H	M4 Pink T	Total score	Comment
48hr	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
	9						
	10						
	11						
	12						
	13						
	14						
	15						
	16						
	17						

Data analysis for the consistency of scorers:

The scores from independent inspectors for each dilution are collected. Consistency between inspectors is assessed. If less than 60% of mice are scored identically and / or more than 4% of the mice have a score difference of 2 or more then the data should be referred to a statistician for a more detailed consideration of the differences between inspectors.

Data analysis for potency:

Reference concentrations should be adjusted for any difference between the actual assigned value and the assumed value of 500 or 100 U/vial (e.g. Actual assigned value divided by assumed value of 500 or 100U X Assumed dilution concentration).

The mean score for each cage is calculated and dose response curves plotted. Activity of the test sample is estimated by comparing with the response obtained with the reference standard. Using the linear region of the dose response curve, a parallel line analysis is performed and potency of test sample calculated relative to the reference standard. Product specific standard of defined activity is included in each assay.